Biochemical Pharmacology, Vol. 28, pp. 1505–1511.
Pergamon Press Ltd., 1979. Printed in Great Britain.

MUSCARINIC ACETYLCHOLINE RECEPTORS IN CAT IRIS

YOEL KLOOG*, DAN I. SACHS†, AMOS D. KORCZYN‡, DAVID S. HERON‡ and MORDECHAI SOKOLOVSKY*,§,¶

Department of Biochemistry, The George S. Wise Faculty of Life Sciences*; Department of Physiology and Pharmacology‡ and from the Maurice and Gabriella Goldschleger Eye Institute, the Chaim Sheba Medical Center§, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv; Department of Ophthalmology, Asaf Harofe Hospital, Zerifin, Israel†

(*Received* 7 *August* 1978; *accepted* 30 *November* 1978)

Abstract—The binding of tritium labeled *N*-methyl-4-piperidylbenzilate([^{3}H]-4-NMPB) to fractions obtained from cat irides was investigated. The binding of this highly potent muscarinic antagonist to the cat iris consisted of two components: (a) high affinity binding sites with low capacity and (b) low affinity binding sites with high capacity. By a simple fractionation procedure those two components were reasonably separated, and a relatively pure fraction with high affinity binding sites was obtained. The high affinity binding sites are attributed to the muscarinic receptor sites, since they showed characteristic behaviour: saturability, high affinity, reduction of the binding by muscarinic ligands, and no effect on binding by non-muscarinic ligands (at a pharmacological range of concentration). Hill coefficients for the antagonists were approximately 1, and for the agonists less than 1. The dissociation constant of [^{3}H]-4-NMPB (0.4–0.6 nM) is similar to that found in albino rabbit irides and in mouse brain. Furthermore, the capacity of the high affinity binding sites (468 fmole/iris) was similar to that found in albino rabbit irides (447 fmole/iris) and in pigmented rabbits irides (512 fmole/iris). According to these findings, the low affinity binding sites cannot be related to the muscarinic receptor and are probably related to the presence of abundant melanin pigment. The interactions of [^{3}H]-4-NMPB with the components of the cat irides and their possible physiological significance are discussed.

The smooth muscles of the iris are governed by antagonistic adrenergic (dilator) and cholinergic (constrictor) influences. The cholinergic neuromuscular system is well studied [1–3] and can be regarded as a classical example of a muscarinic apparatus. And, indeed, the sphincter responds in a characteristic manner to muscarinic agonists and antagonists: agonists and cholinesterase inhibitors cause miosis [4] while the latter agents cause mydriasis [5].

We have previously characterized the muscarinic receptor in mouse brain [6–8] and albino rabbit irides [9]. Marked differences in the mydriatic effect of muscarinic drugs have been found between pigmented and non-pigmented iris sphincters, propably due to the abundant melanin pigment [10]. The present work was aimed at identifying and characterizing the biochemical nature of the muscarinic receptors in pigmented irides, information that will serve to elucidate the events that occur during drug–iris interaction. We summarize here our studies on the interaction of pigmented rabbits and cat irides with [^{3}H]-4-NMPB, a highly potent and specific muscarinic antagonist [11].

MATERIALS AND METHODS

Materials

N-Methyl-4-piperidyl benzilate (4-NMPB) was prepared and tritium labeled (6 Ci/m-mole) as described elsewhere [12]. The chemical and radiochemical purity was determined by analytical thin layer chromatography (Merck Silica 60 plates) in two solvent systems: n-butanol, acetic acid and water (4:1:1), and chloroform, acetone and diethylamine (5:4:1). [^{3}H]-4-NMPB moved as a single peak, identical to the authentic unlabeled compound in these two systems. The purity was > 97 per cent.

(+)QNB and (−)QNB were prepared as described elsewhere [13]. Other compounds used were as follows: oxotremorine (Aldrich), scopolamine·HBr and pilocarpine (Plantex, Israel), atropine sulfate hydrate, propranolol, (+)-tubocurarine, physostigmine salicylate and arecoline·HCl (Sigma), and acetylcholine perchlorate (BDH).

All other compounds were of the best grade available.

Methods

Cats of both sexes weighing 2.5–4 kg were killed by exposure to pure CO_2 in a closed chamber for 3 min. The irides were quickly removed (within 5 min post-mortem), washed in ice cold Krebs solution, dried on a filter paper and weighed in concert (12–24 irides). The irides were removed by inserting a small pair of forceps through a frontal incision in the cornea and grasping the iris; by gentle manipulation the entire iris was extracted intact. While small pieces of the ciliary body complex may have remained attached to the iris, they would have amounted to only a very small fraction of the total tissue weight and were considered insignificant. The irides were scissor-minced and homogenized in 0.32 M ice-cold sucrose (1 iris/0.75 ml) using an Ultra Turrax homogenizer (set-up 7, 30 sec), and then rehomogenized with a motor-driven perspex pestle (950 r.p.m.) in a glass homogenizer (5 strokes) placed in an ice bucket. The whole homogenate was centrifuged in a clinical

¶ M. Sokolovsky is an established investigator of the Chief Scientist's Bureau, Ministry of Health, Israel.

centrifuge (2 min at half maximal speed) and the supernatant collected. The pellet was rehomogenized as described (0.25 ml per 1 iris) and recentrifuged. This supernatant was added to the former to yield the S_1 fraction, which represents a homogenate of 1 iris/ml (~ 2.7 mg protein/ml), and the pellet P_1 was resuspended in 0.32 M sucrose to yield the original volume.

The S_1 fraction was further centrifuged at 17,500 *g* for 30 min (Sorval RC-2B), and the pellet (P_2) resuspended in the original volume.

The same procedure was used for pigmented rabbits.

A second fraction ($S_{1,2}$) was prepared for binding assays. Cat irides were removed and homogenized as described above, but in 1 M sucrose (1 iris/0.75 ml). The whole homogenate was centrifuged at 9750 *g* for 30 min (Sorval RC-2B) and the supernatant collected. The pellet was rehomogenized (in 0.25 ml 1 M sucrose per original homogenized iris), recentrifuged, and this supernatant added to the former. This yielded the ($S_{1,2}$) fraction.

Binding assay. 100–200 μl of the prepared fractions were incubated at 25° in 2 ml modified Krebs solution (25 mM Tris–HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.1 mM glucose), pH 7.4, containing the labeled ligand alone or with various unlabeled drugs. After various periods of incubation, ice-cold Krebs solution (3 ml) was added and the contents were passed rapidly through a glass filter (GF/C, Whatman 25 mm diameter) by suction. The filters were washed 3 times with 3 portions of ice-cold Krebs solution. All procedures were completed within less than 10 sec. Control binding experiments with increasing amounts of total protein, indicated that, when GF/C filters were loaded with more than 1.1 mg protein, lower binding as judged by the measured radioactivity was observed. Therefore, in all experiments no more than 0.8 mg protein/filter was used.

The filters were placed in vials containing 5 ml of scintillation liquid (33 ml triton X-100, 66 ml toluene, 5.5 g PPO (Packard) and 0.1 g POPOP (Merck)), maintained at 25° for 30 min, and then assayed for radioactivity by liquid scintillation spectrometry (Packard Prias model PL). Corrections for quenching were made using an external standard.

All binding experiments were performed in duplicate together with duplicate samples containing 5×10^{-5} M of unlabeled 4-NMPB or atropine. Specific binding was

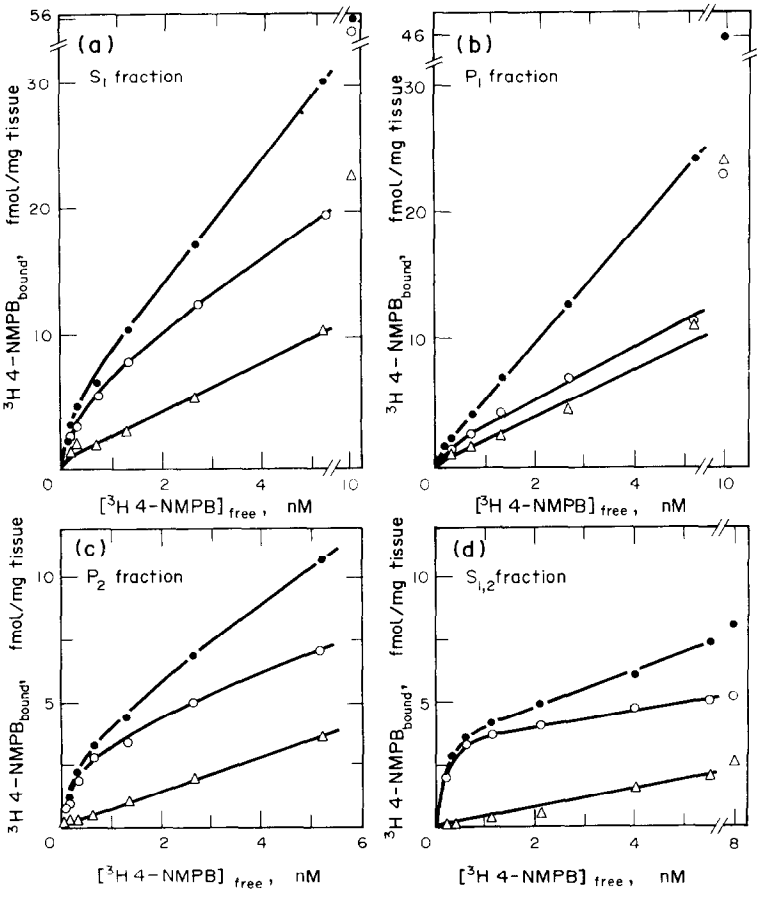

Fig. 1. Binding of [^{3}H]-4-NMPB at 25° as a function of concentration. 0.2 ml samples from the specified fraction were incubated with varying concentrations of [^{3}H] 4 NMPB for 30 min at 25° in 2 ml modified Krebs solution (pH 7.4). ●—●, total binding; △—△, non specific binding (in the presence of 5×10^{-5} M 4-NMPB); ○—○, specific binding. Each experimental point represents the mean of duplicate samples. (a) Binding to the S_1 fraction. (b) Binding to the P_1 fraction. (c) Binding to the P_2 fraction. (d) Binding to the $S_{1,2}$ fraction.

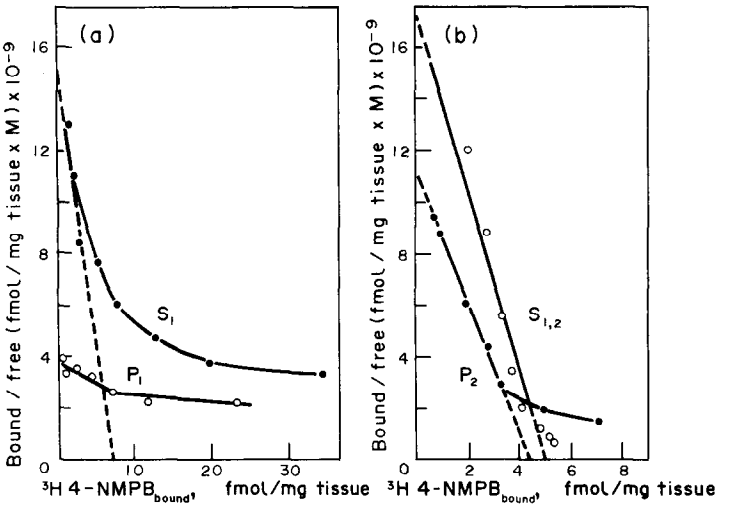

Fig. 2. Specific binding data from Fig. 1 replotted according to Scatchard. (a) for S_1 (●—●) and P_1 (○—○) fraction. (b) for P_2 (●—●) and $S_{1,2}$ (○—○) fractions.

defined as the total binding minus the binding in the presence of excess unlabeled ligand. Lines in Scatchard analysis were fitted by eye.

Protein was determined by the method of Lowry [14], using bovine serum albumin as standard.

RESULTS

The binding of [^{3}H]-4-NMPB at 25° was studied first using the S_1 fraction of the cat iris. The total binding in the S_1 fraction was relatively high and varied with ligand concentration (Fig. 1a, upper curve). In the presence of 50 μM 4-NMPB, the total binding was much lower and varied linearly with ligand concentration (Fig. 1a, lower curve). The value of specific binding was obtained by subtracting the residual binding of [^{3}H]-4-NMPB determined in the presence of excess unlabeled drug (i.e. non-specific binding) from the total value. The specific ligand binding (Fig. 1a) exhibited hyperbolic shape but did not achieve saturation; not even at 40 nM ligand, which is at least 80 times higher a concentration than the K_d value of 4-NMPB from muscarinic receptors in mouse brain [6], guinea pig ileum [11], and albino rabbit iris–ciliary body complex [9]. When the data of Fig. 1a were replotted according to Scatchard [15] (Fig. 2a), they deviated from linearity; thus [^{3}H]-4-NMPB binding could not be related to a single population of binding sites. The data could, however, be accomodated by a two-binding site model consisting of a high affinity binding site (K_d = 0.63 nM, 0.4 pmole/iris) and a low-affinity binding site (K_d > 20 nM). The precise K_d and binding capacity of the latter could not be calculated at the concentration range investigated.

The binding of [^{3}H]-4-NMPB to the P_1 fraction was also very high (Fig. 1b). But when the data of the specific binding in this fraction were replotted according to Scatchard (Fig. 2a), only the low affinity binding site was exhibited. This fraction contains levels of pigment granules at least as high as fraction S_1, but should have much less plasma membranes. The capacity of the low affinity binding sites in P_1 was very high, as it was for the whole homogenate, thereby rendering it impossible to estimate the yield of the high affinity binding sites.

Scatchard plots of [^{3}H]-4-NMPB binding to the S_1 fraction of pigmented rabbit iris–ciliary body complex and albino rabbit iris–ciliary body (Fig. 3), clearly demonstrate that the low affinity binding sites exist only

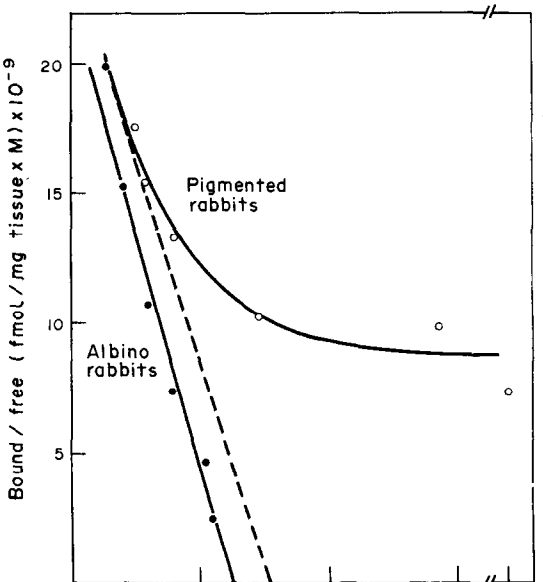

Fig. 3. Scatchard plot for [^{3}H]-4-NMPB binding to albino and pigmented rabbit irides. 0.2 ml S_1 fraction samples were incubated with varying concentrations of [^{3}H]-4-NMPB for 30 min in 2 ml modified Krebs solution (pH 7.4). Binding was assayed in duplicate samples together with duplicate samples containing 5 × 10^{-5} M atropine, and specific binding was determined as described in "Methods". Data were replotted according to Scatchard for pigmented (○—○) and albino (●—●) rabbit irides.

Table 1. Binding capacities and dissociation constants of [^{3}H]-4-NMPB in irides of cats and rabbits

Species	Fraction	B_{max}* fmole/mg tissue	B_{max}* fmole/whole iris	K_d* nM
Cats	S_1	5.7 ± 1.8	468	0.63 ± 0.11
Cats	P_2	4.4 ± 1.1	—	0.41 ± 0.14
Cats	$S_{1,2}$	4.5 ± 1.2	—	0.53 ± 0.20
Pigmented rabbits	S_1	12.8 ± 2.0	512	0.55 ± 0.20
Albino rabbits	S_1	15.7 ± 1.7	447	0.45 ± 0.12

The binding capacities and the K_d values represent the mean of at least three experiments as described in Fig. 1.

* B_{max} and K_d were calculated from the extrapolation values of the Scatchard plots and represent the high affinity binding sites only.

in the former, while the high affinity binding sites exist in both. The K_d values and the binding capacities of the high affinity binding sites are similar for both species (Table 1).

The K_d values of the high-affinity binding sites in cat irides and in pigmented and unpigmented rabbits (Table 1) are very close to those obtained for 4-NMPB in other tissues. In addition, the binding capacity of the various irides is similar. These data led us to conclude that the high-affinity binding site may represent the muscarinic acetylcholine receptor. According to these criteria, the low-affinity binding site could not be related to the muscarinic receptor; the most logical candidate for it was the melanin pigment, since it has been shown that melanin binds *inter alia* muscarinic drugs [16].

In order to further characterize the interaction of [^{3}H]-4-NMPB with the cat iris homogenate, binding studies were performed using the P_2 fraction. As shown in Fig. 1c, the total binding of [^{3}H]-4-NMPB to the P_2 fraction was much lower than that observed for the S_1 fraction. The same was true for non-specific binding. The specific binding exhibited a hyperbolic shape and binding appeared to reach saturation. When the data were replotted according to Scatchard (Fig. 2b) they deviated from linearity in most of the cases. In the P_2 fraction (Fig. 2b) most of the binding could be attributed to the high affinity site (K_d = 0.41 nM). The yield of high affinity binding sites in P_2 fractions relative to S_1 fractions averaged 70 per cent.

Because of the variability of the results with the P_2 fraction, and its relatively low number of high affinity binding sites, another approach was used. In preliminary experiments using a sucrose gradient (0–60 per cent), most of the pigment was found to be concentrated in the 60 per cent fraction of the gradient, and in a narrow band close to the top, with different bands of membranes in between. Based on this finding we decided to homogenize the irides in 1 M sucrose and to use the supernatant ($S_{1,2}$) for binding assays.

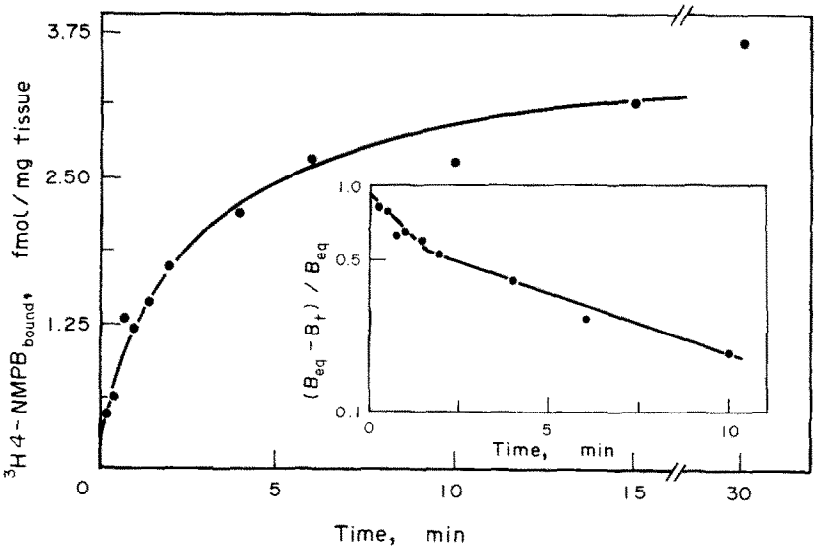

Fig. 4. Time course of [^{3}H]-4-NMPB binding at 25°. $S_{1,2}$ samples (0.2 ml) were incubated in 2 ml modified Krebs solution with [^{3}H]-4-NMPB for various periods of time. Specific binding was determined as described in "Methods". Each experimental point represents the mean value of duplicate samples. Insert: same data plotted according to equation 1.

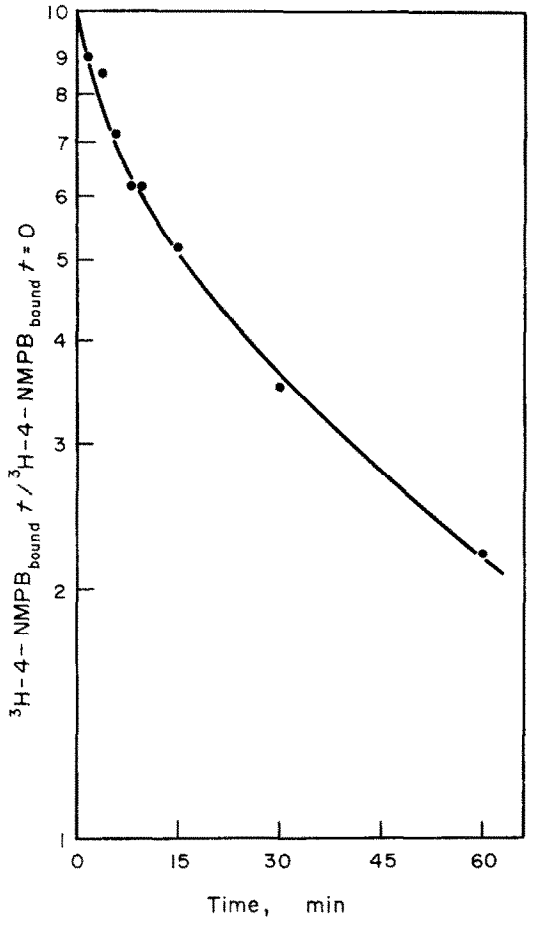

Fig. 5. Dissociation of [^{3}H]-4-NMPB at 25°. $S_{1,2}$ samples (0.2 ml) were incubated for 30 min with 5 nM [^{3}H]-4-NMPB in 2 ml modified Krebs solution. 5×10^{-5} M 4-NMPB was then added and the samples were filtered immediately (zero time) and at times indicated. Specific [^{3}H]-4-NMPB was determined as described in "Methods". Each point represents the mean value of 2 duplicate determinations. ([^{3}H]-4-NMPB bound)$_{t=0}$ was 4.1 fmoles/mg tissue.

Figure 1d represents the binding of [^{3}H]-4-NMPB to $S_{1,2}$ fraction. Specific ligand binding exhibited the hyperbolic shape typical of saturation phenomena (half saturation at 0.5 nM ligand). Non-specific binding was much lower and varied linearly with ligand concentration. When the data in Fig. 1d were replotted according to Scatchard, they exhibited only a slight deviation from linearity (Fig. 2b). The high affinity site ($K_d = 0.53$ nM) in this fraction predominated, and these results were highly reproducible. Thus, further characterization of [^{3}H]-4-NMPB–receptor interaction was performed with $S_{1,2}$ fraction.

Figure 4 shows the time course of association of [^{3}H]-4-NMPB at 2.0 nM, corrected for non-specific binding. Half-saturation was achieved in 1.8 min and maximum binding in approximately 20 min. The kinetic data were replotted according to the integrated rate equation for bimolecular reversible reaction, as discussed by Galper *et al.* [17]:

$$\ln[\text{Beq-B}_t/\text{Beq}] = (k_1[\text{4-NMPB}]-k_{-1})t \qquad (1)$$

where Beq and B_t are the concentration of 4-NMPB–receptor complexes at equilibrium and at time t respectively. The plot deviated from linearity (Fig. 4, insert), indicating that the reaction is not of a simple bimolecular nature.

Similarly, the dissociation of [^{3}H]-4-NMPB–receptor complex, measured by an isotopic dilution technique, exhibited biphasic kinetics (Fig. 5).

To establish the specificity of [^{3}H]-4-NMPB binding in the $S_{1,2}$ fraction of the iris, competition experiments were carried out with various unlabeled ligands (Fig. 6a). The potent muscarinic antagonists (−)QNB, scopolamine and atropine inhibited 50 per cent of the [^{3}H]-4-NMPB binding at the nM range, while the agonist oxotremorine inhibited 50 per cent at 1.6 μM. Non-muscarinic drugs—(+)-tubocurarine, propranolol, haloperidol and phentolamine—at a concentration of 10 μM did not affect the binding. The K_d values for the muscarinic antagonists, as well as the I_{50} values of the agonists (Table 2), correlate well with their known pharmacological potencies [4, 11]. The (−) isomer of QNB is 50 times more potent than the (+) isomer, thus establishing stereospecificity. This is in agreement with the known pharmacological activity of QNB's isomers [13]. Thus, the competition experiments demonstrate that in cat iris homogenate, like in the mouse brain [6], [^{3}H]-4-NMPB binds specifically to muscarinic acetylcholine receptors. Hill plots of the competition curves (Fig. 6b) yielded Hill coefficients close to 1 for all antagonists tested and less than 1 for the agonists (Table 2). Such a pattern of muscarinic binding has been reported previously for several preparations [17–19].

DISCUSSION

The identification of muscarinic acetylcholine receptors in the cat iris using the tritiated highly potent

Table 2. Binding constants and Hill coefficients* (h) for muscarinic ligand as determined in the cat iris by competition with [^{3}H]-4-NMPB

Drug	I_{50}†	K_d(M)‡	h
(−)QNB	1.0×10^{-9}	3.3×10^{-10}	1.02
(+)QNB	2.0×10^{-8}	6.6×10^{-9}	1.05
Scopolamine	3.0×10^{-9}	1.0×10^{-9}	1.04
Atropine	5.6×10^{-9}	1.8×10^{-9}	1.05
Oxotremorine	2.6×10^{-6}	—	0.88
Acetylcholine§	5.5×10^{-6}	—	0.71
Arecoline	1.7×10^{-5}	—	0.82
Pilocarpine	1.7×10^{-5}	—	0.80

* Values given are the means of three separate experiments similar to that in Fig. 4, each performed in duplicate, whose results varied by less than 30 per cent.

† The concentration causing 50 per cent reduction in binding of [^{3}H]-4-NMPB (1.0 nM).

‡ K_d values for antagonists were calculated assuming a simple competitive interaction between [^{3}H]-4-NMPB and the competing ligand, as described elsewhere [6]. Values for K_d for agonists depend on the mechanism responsible for the low Hill coefficients and cannot be calculated from these data.

§ In the presence of 10 μM physostigmine.

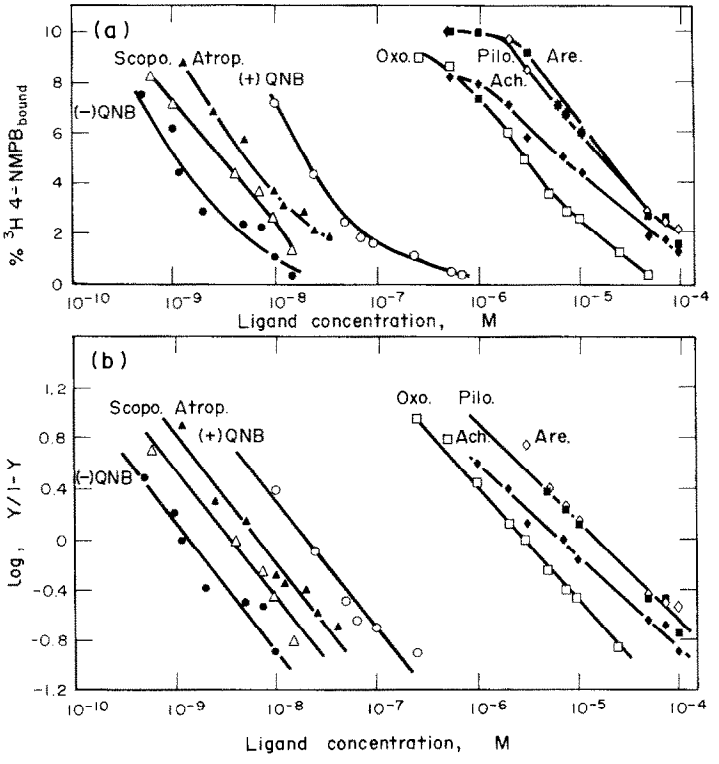

Fig. 6. Competition binding for muscarinic drugs. (a) $S_{1,2}$ samples (0.2 ml) were incubated at 25° for 30 min in 2 ml modified Krebs solution containing 1.0 nM [^{3}H]-4-NMPB and the concentrations of unlabeled ligands indicated. Specific binding was determined as described in "Methods". Each point represents the average of two duplicate determinations. ●—●, (−)QNB; △—△, scopolamine; ▲—▲, atropine; ○—○, (+)QNB; — —, oxotremorine; ◆—◆, acetylcholine; ■—■, pilocarpine and ◇···◇ arecoline. (b) Hill plot of the same data. Y is the fractional binding of [^{3}H]-4-NMPB.

muscarinic antagonist 4-NMPB was complicated by the strong interference of a high degree of binding to non-receptor material which is part of the general category of "non-specific binding". This problem was not unexpected, since Salazar and Patil had shown high binding capacity of [^{3}H]-atropine to melanin [10]. Moreover, Cuatrecasas [20] pointed out many examples of relatively high affinity and highly specific binding which are biological "accidents" rather than meaningful recognition phenomena. The case of the cat iris seemed to be one of these biological "accidents". Fortunately, we had a number of factors which proved to be distinguishing between specific and non-specific binding. These are described below:

(1) The affinity constants of muscarinic antagonists to the receptor in different tissues and animals are very similar. Although slight differences may exist [21], the binding constants generally do not differ by a factor of more than 3 or 4. The binding constants of 4-NMPB to the muscarinic receptor in mouse brain homogenate have been determined [6], and are similar to those of 4-NMPB in albino rabbit iris–ciliary body complex [9] and in guinea pig ileum [11]. Accordingly, only the high affinity binding site detected in cat iris homogenate could be related to the muscarinic acetylcholine receptor (see Table 1).

(2) In general receptors are present in extremely small quantities in physiological membranes. This was confirmed for muscarinic receptor capacities in, among others, chicken [17] and rat hearts [22] and in albino rabbit iris–ciliary body complex [9]; a much higher capacity exists in guinea pig ileum [23], but this seems to be an exception. However, the muscarinic receptor capacity in the albino rabbit iris–ciliary body coincides very well with the high affinity binding site capacity of the pigmented rabbits iris–ciliary body complex and of the cat iris (Table 1). This strongly suggests that only the high affinity binding site in cats and pigmented rabbits irides can be related to the muscarinic receptor.

(3) It has been shown in many preparations, including brain [24] and smooth muscle [25], that the membrane-bound muscarinic receptors have a typical distribution in homogenate fractions. Thus, binding in the S_1 fraction (1000 *g* or less) represents 70–80 per cent of the total homogenate binding, and further centrifugation (17,500 *g* or more) yields most of the receptor binding sites in the pellet (P_2). A similar distribution of the high affinity binding sites in the cat iris was found here. Although it was difficult to demonstrate the high-affinity binding sites in the whole homogenate and P_1 fraction, this site was clearly detected in the S_1 and P_1 fractions. On the other hand the low-affinity binding sites were found in the P_1 as well as in the S_1 fractions. In this regard it is pertinent that muscarinic receptors were demonstrated in 1 M or lower fractions of sucrose gradients [26]; the existence of the [^{3}H]-4-NMPB high-affinity binding sites in the supernatant of the 1 M homogenate ($S_{1,2}$) is consistent with these findings.

Fortunately the low-affinity binding sites pelleted and enabled us to further characterize the high-affinity binding of [^{3}H]-4-NMPB.

The binding of [^{3}H]-4-NMPB to the $S_{1,2}$ fraction of cat iris homogenates exhibits all the characteristics typical for binding to the muscarinic acetylcholine receptor. The binding of the ligand was saturable and high affinity, and unlabeled muscarinic ligands reduced the binding of the labeled drug (Fig. 6a) whereas non-muscarinic drugs did not affect binding. The relative potency of the muscarinic drugs in competing with [^{3}H]-4-NMPB binding (Table 2) correlates well with their pharmacological potencies [4, 11]. Moreover, the typically low Hill coefficient for agonists observed in other tissues [9, 17–19] was established also in the cat iris (Table 2), as were the biphasic association and dissociation kinetics of antagonists binding (Figs. 3 and 4) phenomena observed for [^{3}H]-4-NMPB binding in mouse brain homogenate [6], in albino rabbit iris–ciliary body complex homogenate [9], and for [^{3}H]QNB binding in chicken heart [17] and retina [19]. This biphasic kinetics can be explained by the isomerization process of the receptor–ligand complex, as was previously suggested for [^{3}H]-4-NMPB–receptor interaction in mouse brain [6], and for [^{3}H]QNB–receptor interaction in chicken heart [17]. That is, the association reaction: $R + L \rightleftarrows RL$ is followed by a slow isomerization step: $RL \rightleftarrows R^xL$.

Evaluation of the rate constants of these reactions awaits more detailed kinetic data. However, as an initial approximation, the association rate constant for the $R + L \rightleftarrows RL$ reaction was estimated from the initial rate of binding (Fig. 4) to be $2.7 \times 10^8\ M^{-1}min^{-1}$. The half-time for dissociation (Fig. 5) of [^{3}H]-4-NMPB–receptor complex is about 15 min. These kinetic values are very similar to those of [^{3}H]-4-NMPB in mouse brain [6] and albino rabbit iris–ciliary body complex homogenates [9], and clearly demonstrates the fast "on" and "off" rates.

The high capacity of low-affinity binding sites for [^{3}H]-4-NMPB in the cat and pigmented rabbit irides—binding sites that do not exist in the albino rabbits—suggests that these binding sites are the melanin pigments or associated factors. This conclusion is supported by the known phenomena of drugs binding to pigments. Moreover, Shimada *et al.* showed binding of another muscarinic antagonist [^{3}H]atropine—to "artificial" melanin [16]. Salazar and Patil [10] showed a greater retention of [^{3}H]atropine in pigmented rabbit irides than in unpigmented irides, and related this to the prolonged mydriatic effect of atropine in pigmented rabbits. Our data support their conclusion, since the similar rates of [^{3}H]-4-NMPB–receptor interaction in unpigmented rabbits and in cat irides indicate that the longer mydriatic effect in pigmented irides cannot be attributed to differences in the drug–receptor interaction. Furthermore, the existence of low-affinity binding sites for [^{3}H]-4-NMPB in pigmented irides only suggest that 4-NMPB, like atropine, can bind to the pigments.

The binding of muscarinic antagonists by pigments is not likely to be the sole explanation for their prolonged mydriatic effect in general. Even in albino rabbits [10] and mice [27], the mydriatic effect is much longer then the dissociation rate from the receptor. However, it has been shown that the higher the antimuscarinic activity of a drug, the longer the duration of the mydriasis [27]. This potency–duration profile has been also confirmed in binding studies of labeled muscarinic antagonists [7] and suggests that, in a given species, the events at the receptor sites determine the relative mydriatic potency in a series of drugs.

Acknowledgement—The skilful technical assistance of Mrs. Ronit Galron is gratefully acknowledged.

REFERENCES

1. S. Consolo, S. Garattini, H. Ladinsky and H. Thoenen, *J. Physiol., Lond.* **220**, 639 (1972).
2. R. Lund-Karlsen and F. Fonnum, *Acta Pharmac. Tox.* **28**, 299 (1976).
3. J. S. Mindel and T. W. Mittag, *Invest. Ophthal.* **15**, 803 (1976).
4. S. Maayani, G. Treister and M. Sokolovsky, *Invest. Ophthal.* **14**, 232 (1975).
5. G. Triester, S. Maayani and M. Sokolovsky, *Invest. Ophthal.* **14**, 159 (1975).
6. Y. Kloog and M. Sokolovsky, *Brain Res.* **144**, 31 (1978).
7. Y. Kloog and M. Sokolovsky, *Biochem. biophys. Res. Commun.* **81**, 710 (1978).
8. Y. Kloog and M. Sokolovsky, *Brain Res.* **134**, 167 (1977).
9. Y. Kloog, D. I. Sachs, D. S. Heron, A. D. Korczyn and M. Sokolovsky, *Molec. Pharmac.*, in press.
10. M. Salazar and P. N. Patil, *Invest. Ophthal.* **15**, 671 (1976).
11. M. Rehavi, B. Yaavetz, Y. Kloog, S. Maayani and M. Sokolovsky, *Biochem. Pharmac.* **27**, 1117 (1978).
12. A. Kalir, S. Maayani, M. Rehavi, R. Elkavets, I. Pri-Bar, U. Buchman and M. Sokolovsky, *Eur. J. med. Chem.* **13**, 17 (1978).
13. M. Rehavi, S. Maayani and M. Sokolovsky, *Life Sci.* **21**, 1293 (1977).
14. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
16. K. Shimada, R. Baweja, T. D. Sokolosky and P. N. Patil, *J. Pharm. Sci.* **66**, 1057 (1976).
17. J. B. Galper, W. Klein and W. Catterall, *J. biol. Chem.* **252**, 8692 (1977).
18. N. J. M. Birdsal and E. C. Hulme, *J. Neurochem.* **27**, 7 (1976).
19. H. Sugiama, M. P. Daniels and M. Nirenberg, *Proc. natn. Acad. Sci. U.S.A.* **74**, 5524 (1977).
20. P. Cuatrecases and M. D. Hollenberg, *Biochem. biophys. Res. Commun.* **62**, 31 (1975).
21. Y. Kloog, Y. Egozi and M. Sokolovsky, *Molec. Pharmac.*, in press.
22. D. Cavey, J. P. Vincent and M. Lazdunsky, *FEBS Lett.* **84**, 110 (1977).
23. H. I. Yamamura and S. H. Snyder, *Molec. Pharmac.* **10**, 861 (1974).
24. P. Alberts and T. Bartfai, *J. biol. Chem.* **251**, 1543 (1976).
25. A. J. Beld and E. J. Ariens, *Eur. J. Pharmac.* **25**, 203 (1972).
26. T. Bartfai, P. Berg, M. Schultzberg and E. Heilbronn, *Biochim. biophys. Acta* **426**, 186 (1976).
27. I. D. Inch, D. M. Green and P. B. J. Thompson, *J. Pharm. Pharmac.* **25**, 359 (1973).